Rejection under 35 U.S.C. §102(b)

The Examiner has rejected Claims 10, 30-32, and 34 under 35 U.S.C. §102(b) as being anticipated by Johansson et al. (Eur Respir. J. (1994) 7: 372-391). More specifically, the Examiner believes that since the Johansson et al reference states that "[h]uman surfactant, isolated from amniotic fluid by sucrose gradient centrifugation, as described by HALLMAN et al. [293], has been used mainly in Finland and California. It contains, apart from lipids, approximately 6% proteins, including hydrophilic (SP-A, SP-D, and nonsurfactant proteins)...", it meets all of the limitations of the claims.

However, amended Claim 10 is drawn to a "A pharmaceutical composition effective in treating pulmonary disease in mammals comprising: isolated, purified, or recombinant SP-D protein". Johansson et al. teaches human surfactant isolated from amniotic fluid. Such isolated surfactant contains numerous proteins, including surfactant proteins, non-surfactant proteins and lipids. *See, Johansson*, at page 381, second column, second paragraph. Accordingly, Johansson does not disclose "isolated, purified or recombinant SP-D protein," and therefore, cannot anticipate the presently claimed invention.

Rejection under 35 U.S.C. §102(b)

The Examiner has rejected Claims 10, 30-32, and 34 under 35 U.S.C. §102(b) as being anticipated by Jobe et al. (Am Rev. Respir. Dis. (1987) 136: 1256-1275). Claim 10 and the dependent claims are drawn to a "pharmaceutical composition effective in reducing pulmonary inflammation in mammals comprising: isolated, purified, or recombinant SP-D protein". The Examiner believes that Jobe et al teach that human surfactant from amniotic fluid (which inherently contains SP-D) has been used in clinical trials for the treatment of RDS. However, Jobe, like Johansson, only teaches human surfactant isolated from amniotic fluid. As discussed above, such surfactant contains a complex mixture of proteins and other materials. Thus, Jobe does not comprise "isolated, purified, or recombinant SP-D protein," and does not anticipate the presently claimed invention.

Rejection under 35 U.S.C. §103(a)

The Examiner has rejected Claims 10, and 29-34 under 35 U.S.C. §103(a) as being unpatentable over Johansson et al. (Eur Respir. J. (1994) 7: 372-391) in view of Jain-Vora. More specifically, the Examiner believes that the Johansson et al. reference teaches a pharmaceutical composition comprising SP-A, SP-B, SP-C, and SP-D and that SP-D and SP-A play a role in the host defense system of the lung. The Jain-Vora reference was cited specifically in relation to the limitations recited in Claims 29-33.

Prima facia obviousness requires the inclusion of all claimed elements in the combination of references as well as a motivation to combine the elements in the claimed manner. All of the claimed elements are not taught in the combination of references, because neither of the references teaches an isolated, purified, or recombinant SP-D protein, but only teach SP-D in admixture with other surfactant proteins in amniotic fluid or surfactant. In addition, there would be no motivation to use isolated, purified, or recombinant SP-D as a pharmaceutical for the following reasons:

As noted above, the presently claimed invention is a pharmaceutical preparation comprising an isolated, purified, or recombinant SP-D protein. One of ordinary skill in the art would not be motivated to make such a preparation from the isolated surfactant of Johansson, because such an individual would know that a pharmaceutically acceptable SP-D preparation with stability, sterility, and which is homogeneous could not be made from whole or isolated surfactant. Surfactant contains numerous proteins - perhaps hundreds - which have antigenic potential and multiple actions. Thus, Johansson's disclosure of the use of isolated surfactant would not motivate one of ordinary skill to isolate any one of the individual ingredients of the surfactant for pharmaceutical use in surfactant replacement or any other application.

Johansson et al. do make reference to SP-D individually. However, this disclosure in Johansson would also fail to motivate one of ordinary skill in the art to isolate or purify SP-D protein for pharmaceutical use. Johanssonn et al. cite that "SP-D enhance[s] the production of oxygen radicals by the same cells (alveolar macrophages)". *See*, page 377, first column, first paragraph. However, as explained in the enclosed Declaration by Jeffrey Whitsett, one of skill in the art knows that the production of oxygen radicals in alveolar macrophages is part of the inflammatory process in response to an invading organism in order to remove that organism from the lungs. As explained in greater detail below, one of ordinary skill in the art would view the induction of the inflammatory process as detrimental in the pharmaceutical treatment of

pulmonary diseases. Thus, armed with *the knowledge given by Johansson et al. that SP-D is involved in the inflammatory process*, one of skill in the art would not be motivated to isolate or purify SP-D protein to produce a pharmaceutical preparation for treatment of lung inflammation.

Notwithstanding the findings reported in Johansson, the present inventors have discovered that SP-D protein has the effect of *reducing* inflammation. Thus, Johansson actually teaches away from the present invention in teaching that SP-D protein has activity in the production of oxygen radicals in alveolar macrophages. Accordingly, it is improper to base an obviousness rejection on the teachings of Johansson. *See, e.g., In re Zurko*, 42 U.S.P.Q.2d 1476 (Fed. Cir. 1997).

When treating diseases such as emphysema (or other non-infectious diseases of the lungs), one would not want an inflammatory pharmaceutical, because the pathogenesis of emphysema involves oxidant injury and inflammation and is worsened by oxidant production. The findings shown in the Specification and the Declaration by Jeffery Whitsett are just the opposite. They show that SP-D suppresses oxidant injury by interacting with the macrophages to inhibit oxidant production and inflammatory signaling. SP-D enhances host defense by macrophages by modifying the cellular response to suppress the inflammatory response - not to stimulate it. Therefore, one of skill in the art would not be motivated to isolate SP-D and produce a treatment for non-infectious diseases.

Similarly, one of skill in the art would not be motivated to produce a pharmaceutical for treating infectious diseases of the lungs with the knowledge that SP-D enhances oxygen radicals in alveolar macrophages. Although at first glance, one might expect that increased oxygen radicals could enhance the host immune response and remove the infectious agents, one of skill in the art would not be trying to remove the infectious agent. This is because the pathogenesis of infectious diseases of the lungs is predominantly due to two aspects, 1. the infection and 2. the host inflammatory response. The first aspect is easily treated with antibiotics or by the host immune response. However, the second aspect, the host inflammatory response remains, even after clearance of the infectious agent, and continues to cause damage. Thus, based on the knowledge that SP-D enhances oxygen radicals in alveolar macrophages, one of skill in the art would not be motivated to treat infectious diseases of the lungs with SP-D because SP-D would enhance the damage due to the inflammatory response.

In addition, as stated in the Declaration by Jeffery Whitsett, the results that Johansson et al discuss are likely a result of contamination of SP-D with LPS (endotoxin) or a pathogen and this leads to an incorrect conclusion.

In conclusion, the statement in Johansson et al that SP-D enhances oxygen radicals in alveolar macrophages would not motivate one of skill in the art to produce a pharmaceutical preparation of SP-D.

Secondly, even if the statements in Johansson et al. had motivated one of skill in the art to try to produce a pharmaceutical preparation of SP-D, there is no reasonable likelihood of success. This is because, the studies in Johansson et al which showed that SP-D enhanced oxygen radicals in alveolar macrophages (thus, increasing inflammation) were done *in vitro* with alveolar macrophages in tissue culture. There is no reasonable likelihood of success, because when going from *in vitro* to *in vivo* studies, one can not predict what might happen and, in fact, as explained in the Specification on page 20, line 18, when *in vivo* studies were done in a mouse model using SP-D, the opposite effect was observed. SP-D reduced inflammation. Additional support for this can be found in Yoshida, et al. *J. Immunol.* 166. 75 14, 2001 and the Declaration by Jeffrey Whitsett.

Further, there is no reasonable likelihood of success because the *in vitro* experiments by Van Ivaarden, et al. which showed an inflammatory effect of SP-D were most likely due to a problem with the purification of SP-D historically. As stated in the Declaration by Jeffery Whitsett, the purified SP-D historically contained LPS (endotoxin) or other inflammatory stimuli. Newer methods of purification have allowed for a clear understanding of the true actions of purified SP-D.

Claims 29 and 33 additionally recite the presence of IL-4 in the preparation. Jain-Vora et al teaches that IL-4 is involved in bacterial host defense systems by enhancing pulmonary clearance of bacteria. Thus, the Examiner believes that one of skill in the art would have been motivated to improve the pharmaceutical composition of Johannson by adding IL-4. However, these claims are patentable because they include all of the limitations of the claims from which they depend, as discussed above.

Conclusion

Should there be any questions regarding the above-identified patent application, the Examiner is respectfully requested to contact the undersigned at the telephone number appearing below. Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 3/8/02

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

CLAIM AMENDMENTS

10. **(Amended)** A pharmaceutical composition effective in [treating] reducing pulmonary [disease] inflammation in mammals comprising: isolated, purified, or recombinant SP-D protein in admixture with a pharmaceutically acceptable excipient.

Surfactant Protein D Regulates NF-κB and Matrix Metalloproteinase Production in Alveolar Macrophages via Oxidant-Sensitive Pathways[1]

Mitsuhiro Yoshida, Thomas R. Korfhagen, and Jeffrey A. Whitsett[2]

Targeted ablation of the surfactant protein D (SP-D) gene caused progressive pulmonary emphysema associated with pulmonary infiltration by foamy alveolar macrophages (AMs), increased hydrogen peroxide production, and matrix metalloproteinase (MMP)-2, -9, and -12 expression. In the present study, the mechanisms by which SP-D influences macrophage MMP activity were assessed in AMs from SP-D$^{-/-}$ mice. Tissue lipid peroxides and reactive carbonyls were increased in lungs of SP-D$^{-/-}$ mice, indicating oxidative stress. Immunohistochemical staining of AMs from SP-D$^{-/-}$ mice demonstrated that NF-κB was highly expressed and translocated to the nucleus. Increased NF-κB binding was detected by EMSA in nuclear extracts of AMs isolated from SP-D$^{-/-}$ mice. Antioxidants *N*-acetylcysteine and pyrrolidine dithiocarbamate inhibited MMP production by AMs from SP-D$^{-/-}$ mice. To assess whether increased oxidant production influenced NF-κB activation and production of MMP-2 and -9, AMs from SP-D$^{-/-}$ mice were treated with the NADPH oxidase inhibitors diphenylene iodonium chloride and apocynin. Inhibition of NADPH oxidase suppressed NF-κB binding by nuclear extracts and decreased production of MMP-2 and 9 in AMs from SP-D$^{-/-}$ mice. SN-50, a synthetic NF-κB-inhibitory peptide, decreased MMP production by AMs from SP-D$^{-/-}$ mice. Oxidant production and reactive oxygen species were increased in lungs of SP-D$^{-/-}$ mice, in turn activating NF-κB and MMP expression. SP-D plays an unexpected inhibitory role in the regulation of NF-κB in AMs. *The Journal of Immunology,* **2001, 166: 7514–7519.**

Surfactant protein D (SP-D)[3] is a 43-kDa member of a family of collagenous carbohydrate binding proteins (C-type lectins), termed the collectins. In the lung, SP-D is synthesized and secreted primarily by Type II cells and other nonciliated respiratory epithelial cells. SP-D monomers are assembled into dodecamers consisting of four homotrimeric subunits (1). Shared structural motifs with other collectins and in vitro studies suggest that SP-D plays a role in innate immunity against various pulmonary pathogens. To evaluate its function in vivo, SP-D-null mice (SP-D$^{-/-}$) were generated (2, 3). Targeted gene inactivation of the SP-D gene in mice caused the accumulation of surfactant phospholipids, emphysema, and increased numbers of lipid-laden, foamy alveolar macrophages (AMs). Emphysema in SP-D$^{-/-}$ mice was associated with increased production of matrix metalloproteinases (MMP)-2, -9, and -12, hydrogen peroxide, and increased proinflammatory cytokine production after pulmonary infection (4, 5). Although focal production of cytokines, MMP, and H_2O_2 may play a role in the development of emphysema in SP-D$^{-/-}$ mice, the mechanisms mediating the generation of these molecules have not been identified.

Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydroxyl radical (OH·), and hydrogen peroxide (H_2O_2) have been implicated in the pathogenesis of several lung diseases associated with oxidative stress, including emphysema, adult respiratory distress syndrome, asthma, and lung fibrosis (6). Although ROS play a critical role in host defense, increased ROS-generated during acute and chronic inflammation can be cytotoxic, causing oxidative damage to various macromolecules, lipid peroxidation, protein cross-linking, protein fragmentation, DNA damage, and strand breaks (7). Oxidative stress has been associated with activation of transcriptional pathways mediating cellular responses to infection and injury. For example, activity of NF-κB and AP-1, were stimulated by oxidative stress (8). Binding sites for NF-κB and AP-1 were identified in the promoters of numerous genes, including proinflammatory cytokines (9). Likewise, *cis*-acting elements binding NF-κB and/or AP-1 were present in the promoter regions of the MMP-2, -9, and -12 genes (10–13). Therefore, we hypothesized that ROS, generated by AMs in SP-D$^{-/-}$ mice, might activate redox-sensitive transcription factors, causing increased expression of the MMP. In the present study, increased ROS were demonstrated in lungs of SP-D$^{-/-}$ mice. MMP-2 and -9 production by AMs from SP-D$^{-/-}$ mice was stimulated by oxidant-sensitive pathways, including NF-κB activation.

Materials and Methods

Animals

SP-D$^{-/-}$ mice were generated by targeted gene inactivation (3). SP-D$^{-/-}$ mice survive and breed normally in the vivarium under barrier containment facilities at Children's Hospital Medical Center (Cincinnati, OH). Experimental procedures were reviewed and approved by the Children's Hospital Institutional Animal Care and Use Committee. Swiss black SP-D$^{-/-}$ and age-matched Swiss black SP-D$^{+/+}$ wild-type (WT) mice were mated separately to generate animals for this study. Evidence of viral and bacterial pathogens was not detected in sentinel mice in the colony.

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[3] Abbreviations used in this paper: SP-D, surfactant protein D; AM, alveolar macrophage; MMP, matrix metalloproteinase(s); ROS, reactive oxygen species; WT, wild type; BAL, bronchoalveolar lavage; LPO, lipid hydroperoxide; OHNAH, 3-OH-2-naphthoic acid hydrazine; CDCFH, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; NAC, *N*-acetylcysteine; PDTC, pyrolidine dithiocarbamate; DPI, diphenylene iodonium chloride.

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Bronchoalveolar lavage (BAL) and isolation of AMs

BAL was performed by instilling 10 1-ml aliquots of PBS. BAL fluid from several animals was pooled to provide sufficient numbers of macrophages for each analysis. The lavage was centrifuged at 1200 rpm at 7 min, and pelleted cells were resuspended in serum-free RPMI 1640 medium containing 1% of Nutriodoma (Boehringer Mannheim, Indianapolis, IN), and counted with a hemocytometer. More than 90% of BAL cells were AM in both WT and SP-D$^{-/-}$ mice. For some experiments, AMs were isolated by differential attachment to tissue culture flasks at 37°C. Nonadherent cells then were removed, and fresh, serum-free medium was added. The adherent AMs were maintained in a humidified atmosphere containing 5% CO_2 and 95% air until the end of experiments.

Lipid hydroperoxide (LPO) concentration

LPO was measured in whole lung from SP-D$^{-/-}$ and WT mice with the LPO-586 assay kit (Oxis International, Portland, OR). Lungs were isolated and homogenated with PBS containing 5 mM butylated hydroxytoluene and centrifuged 15,000 rpm for 15 min at 4°C. Supernatants were collected and the content of malonaldehyde and 4-hydroxyalkenals was measured colorimetrically following the manufacturer's procedures.

Histochemical detection of lipid peroxidation-derived carbonyls

Lung sections (10 μm thick) obtained from frozen tissue specimens were exposed for 1 h at 60°C to a 0.1% 3-OH-2-naphthoic acid hydrazine (OHNAH) solution in 50% ethanol containing 5% acetic acid. After the reaction, the sections were washed thoroughly in 50% ethanol and stained for 5–10 min with a 0.1% fast blue B solution in an alcoholic buffer prepared by mixing equal volumes of 100 mM phosphate buffer, pH 7.4, and 95% ethanol. Carbonyls are converted to naphthoic hydrazones by reaction with OHNAH. Coupling with the diazonium salt then yields violet azo dyes (14).

Detection of intracellular ROS

Intracellular ROS accumulation was determined with the fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CDCFH; Molecular Probes, Eugene, OR). AMs were incubated with 10 μM CDCFH for 30 min, rinsed with PBS, and fixed with 4% paraformaldehyde. Fluorescence was observed by fluoromicroscopy with excitation and emission wavelengths of 485 and 530, respectively.

Immunostaining for NF-κB p65

BAL cells were isolated from WT and SP-D$^{-/-}$ mice, cytospun and fixed with cold methanol for 10 min, and washed in PBS. The slides then were incubated at 4°C overnight with a rabbit anti-p65 Ab (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation, the slides were washed in PBS and incubated with FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) as a second Ab, and compared with samples prepared identically without primary Ab.

Treatment of AMs in vitro

AMs from SP-D$^{-/-}$ mice were pooled and placed in culture at a concentration of 5×10^5 cells per well in serum-free RPMI 1640 medium. The AMs were treated with 20 mM *N*-acetylcysteine (NAC), 200 μM pyrolidine dithiocarbamate (PDTC), 1 μM diphenylene iodonium chloride (DPI; Sigma, St. Louis, MO), or 1 mM apocynin (Aldrich, Milwaukee, WI). Cells also were incubated with a 10 μM SN-50 (Calbiochem, La Jolla, CA), an inhibitor of NF-κB nuclear import. After 6 h of incubation, supernatants were removed and the cells were washed and incubated with fresh medium including the reagents for 24 h. At the concentrations used, these agents did not alter macrophage viability, as determined by trypan blue exclusion or lactate dehydrogenase measurement (Roche, Indianapolis, IN). RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM containing 10% FBS, 10 mM HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells (2×10^5) cells in 24-well plates were incubated with or without 10 μM menadione (Sigma) for 24 h.

Gelatin zymography

MMP activities were measured in macrophage-conditioned medium. Proteinases in the conditioned medium were concentrated by incubation with gelatin-agarose beads (Amersham Pharmacia, Arlington Heights, IL) for 2 h at 4°C. The beads were pelleted and washed, and proteinases were eluted by incubation in sample buffer for 45 min at 37°C. The samples then were electrophoresed into 10% Zymogram gelatin gels (NOVEX, San Diego, CA). After electrophoresis, gels were washed twice with 2.5% Trito X-100 (37°C, 30 min) and incubated for 16 h with 40 mM Tris-HCl (p 7.5), 10 mM $CaCl_2$, and 1 μM $ZnCl_2$. Gels were stained with 0.5% (w/ Coomassie blue in 50% methanol and 10% acetic acid for 30 min, the destained. MMP were detected as clear bands against a blue backgroun

Nuclear extract preparation

Nuclear extracts were obtained by using a modified method described pr viously (15). Lavaged cells were lysed with Buffer A (10 mM HEPES, 1 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% Nonidet P-40, 1 mM DT and 0.5 mM PMSF, followed by vortexing to shear the cytoplasmic mem branes. Nuclei were pelleted by centrifugation at 3000 rpm for 3 min at 4° in a microcentrifuge. Nuclear proteins were extracted with high-salt buff C (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1.5 m $MgCl_2$, 420 mM NaCl, 1 mM DTT, and 0.5 mM PMSF) and stored −80°C. Total nuclear protein concentrations were determined by the bici choninic acid method.

EMSA

Activation of transcription factors NF-κB and AP-1 were assessed b EMSA with consensus oligonucleotides of NF-κB (AGT TGA GGG GA TTT CCC AGGC) and AP-1 (CGC TTG ATG AGT CAG CCG GA/ Promega, Madison, WI), respectively. Probes were end-labeled with T polynucleotide kinase in the presence of [γ-^{32}P]ATP. Labeled probes wer purified on a Nick column (Pharmacia, Piscataway, NJ). Nuclear protein (μg) was incubated with labeled probes for 15 min at room temperatur The mixture was electrophoresed and the gel dried and subjected to aut radiography. Band specificity was determined by competition experimen with a molar excess of double-stranded nucleotide probes consisting of a unlabeled NF-κB or AP-1 consensus binding site that was added to nuclea extracts before the addition of labeled probes. Supershift assays for NF-κ proteins also were done with polyclonal Abs obtained from Santa Cru Biotechnology. Specific Abs against p65, p50, or c-*Rel* were incubate with the nuclear extracts for 1 h at 4°C before the labeled probes wer added.

MMP-2 and -9 mRNA by RT-PCR

Total RNA from macrophages was extracted by TRIzol reagent (Life Tech nologies, Gaithersburg, MD) according to the manufacturer's protocol. Re verse transcription was conducted for 45 min at 42°C with oligo(dT) an Moloney murine leukemia virus reverse transcriptase (Life Technologies cDNA were amplified with various primers specific for the cDNA se quences of the following molecules: MMP-2 (5′-TCT GCG GGT TC CTG CGT CCT GTG C-3′, 5′-GTG CCC TGG AAG CGG AAC GG AAC T-3′), MMP-9 (5′-TTC TCT GGA CGT CAA ATG TGG-3′, 5 CAA AGA AGG AGC CCT AGT TCA AGG-3′), β-actin (5′-GTG GG CGC TCT AGG CAC CAA-3′, 5′-CTC TTT GAT GTC ACG CAG GA TTC-3′). The PCR products were electrophoresed in 1% agarose gels an stained with ethidium bromide-stained gels that were imaged by using th Alpha-Imager 2000 Documentation and Analysis Software (Alpha Inno tech, San Leandro, CA).

Statistics

Results are presented as means ± SE. Comparison was made by Student' *t* test. Statistical calculations were performed with the Statview II statistica package (Abacus Concepts, Berkeley, CA). A value of $p < 0.05$ was re garded as significant.

Results

Increased oxidant stress in lungs of SP-D$^{-/-}$ mice

To determine whether oxidant stress was increased in the lungs o SP-D$^{-/-}$ mice, LPO concentrations were assessed. LPO content o lung homogenates was increased significantly in lungs of SP-D$^{-/-}$ compared with those from WT mice (Fig. 1). Histochemical stain ing with OHNAH tetrazolium, a reagent that detects reactive car bonyls, demonstrated increased staining in lung sections from SP D$^{-/-}$ mice. The intensity of OHNAH staining in SP-D$^{-/-}$ mic was not uniform, being most prominent at the sites of foamy mac rophage infiltration (Fig. 2). Intracellular ROS in AMs were de termined by CDCFH, an indicator of intracellular peroxides, in cluding H_2O_2 and lipid peroxides. Increased CDCFH fluorescenc was observed in AMs from SP-D$^{-/-}$ compared with those fron

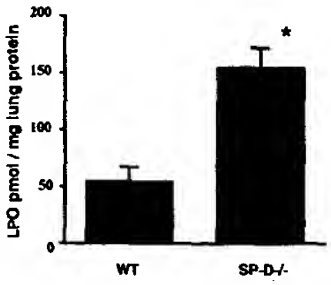

FIGURE 1. Lung lipid hydroperoxidase concentrations are increased in lungs of SP-D$^{-/-}$ mice. Lung tissues from adult WT and SP-D$^{-/-}$ mice were homogenized, and the content of malonaldehyde and 4-hydroxyalkanels measured colorimetrically. LPO was significantly increased in lungs from SP-D$^{-/-}$ mice. Values shown are means ± SE; $n = 5$; *, $p < 0.05$.

control mice (Fig. 3). Taken together with previous findings demonstrating increased hydrogen peroxide production by AMs from SP-D$^{-/-}$ mice, the present data support the concept that oxidative stress is increased in pulmonary tissues in the absence of SP-D.

Activation of NF-κB in SP-D$^{-/-}$ mice

ROS activate redox-sensitive transcription factors, including NF-κB and AP-1 (8). Immunofluorescence staining analysis with anti-NF-κB p65 Ab demonstrated that the p65 subunit of NF-κB was present in the cytoplasm of AM from both WT and SP-D$^{-/-}$ mice (Fig. 4*A*). However, in AMs from SP-D$^{-/-}$ mice, increased staining for NF-κB p65 was observed; furthermore, nuclear staining was markedly increased in AMs from SP-D$^{-/-}$ mice and was almost never detected in AMs from WT mice. NF-κB activity was determined in nuclear extracts from SP-D$^{-/-}$ mice by assessing binding to a consensus NF-κB oligonucleotide in EMSA (Fig. 4*B*). Increased NF-κB binding was observed in nuclear extracts from AMs of SP-D$^{-/-}$ mice. Binding of the nuclear extract to the NF-κB site was inhibited by coincubation with the unlabeled NF-κB oligonucleotide, supporting the specificity of the EMSAs. Likewise, AP-1 binding activity was increased in nuclear extracts from AMs of SP-D$^{-/-}$ mice. Supershift assay for NF-κB showed

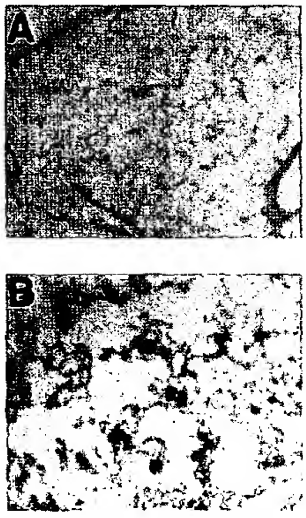

FIGURE 2. Increased reactive carbonyls in lungs of SP-D$^{-/-}$ mice. Frozen sections of lung from WT and SP-D$^{-/-}$ mice were incubated with OHNAH, followed by coupling with diazonium. Reactive carbonyls were observed at the sites of foamy AM infiltration in SP-D$^{-/-}$ (*B*) but not in control mice (*A*). Figures are representative of three separate experiments.

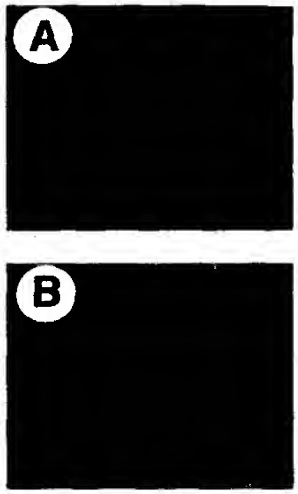

FIGURE 3. Increased intracellular ROS in AMs from SP-D$^{-/-}$ mice. AMs from WT and SP-D$^{-/-}$ mice were incubated with CDCFH for 30 min. Increased fluorescence was observed in AMs from SP-D$^{-/-}$ mice (*B*) compared with those from controls (*A*). Data are representative of three separate experiments.

that the protein/DNA complex contained both components of NF-κB p50 and p65, but not c-*Rel* (Fig. 4*C*).

Antioxidants inhibit MMP expression by AMs from SP-D$^{-/-}$ mice

To determine whether increased oxidant production mediated the expression of MMP by AMs from SP-D$^{-/-}$ mice, the cells were treated with NAC and PDTC, both antioxidant reagents (7, 16). Gelatinolytic activity in the culture medium of untreated and treated cells was analyzed by SDS-PAGE zymography (Fig. 5). Treatment of AMs from SP-D$^{-/-}$ mice with NAC and PDTC reduced gelatinolytic activity consistent with mobility of MMP-2 and -9. Because NADPH oxidases are important sources of ROS in macrophages, we assessed whether ROS generated by NADPH oxidases or other oxidases mediated the increased MMP expression characteristic of SP-D$^{-/-}$ mice. AMs from SP-D$^{-/-}$ mice were incubated with NADPH oxidase inhibitors DPI and apocynin. SDS-PAGE zymography demonstrated that both of the NADPH oxidase inhibitors markedly suppressed MMP enzymatic activity (Fig. 6*A*). Likewise, apocynin reduced MMP-2 and -9 mRNA in cultured AMs from SP-D$^{-/-}$ mice (Fig. 6*B*). Apocynin reduced binding of nuclear extracts from AMs isolated from SP-D$^{-/-}$ mice to a NF-κB oligonucleotide (Fig. 6*C*). Apocynin also decreased DNA binding activity to an AP-1 oligonucleotide (data not shown). AMs were cultured with SN-50, a synthetic inhibitory peptide that blocks nuclear import of NF-κB (17). SN-50 markedly suppressed MMP-2 and -9 production by AM from SP-D$^{-/-}$ mice (Fig. 7).

To assess whether ROS directly stimulated MMP production, RAW 264.7 macrophages were incubated with 10 μM menadione, a ROS generator, for 24 h. MMP-9 production was increased by menadione as assessed by zymography (data not shown).

Discussion

SP-D deficiency caused increased oxidative stress in pulmonary tissues associated with redox-sensitive enhancement of NF-κB activity, and increased MMP production by AMs. Because NF-κB is a critical mediator of transcriptional responses during inflammation, these findings support the concept that SP-D is required for

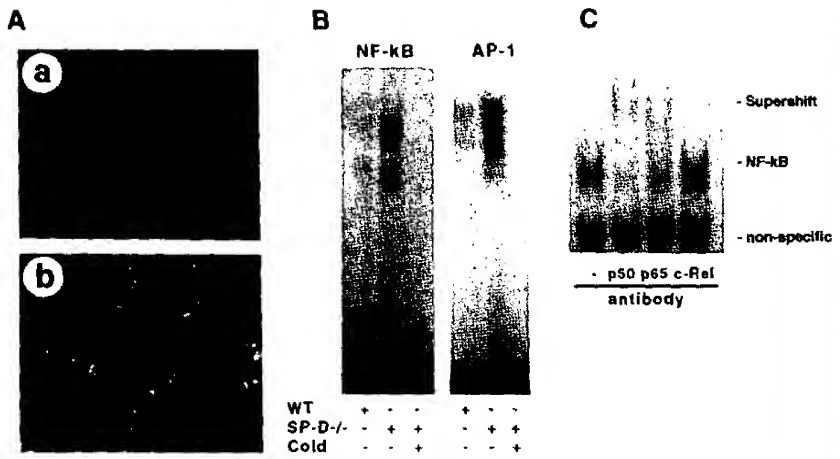

FIGURE 4. NF-κB activation in AMs from SP-$D^{-/-}$ mice. *A*, Immunofluorescence staining for NF-κB p65 in AMs from WT and SP-$D^{-/-}$ mice. Lavaged cells from SP-$D^{-/-}$ mice and age-matched controls were prepared for immunohistochemistry. Intense staining for NF-κB was observed in the cytoplasm and nuclei of AMs from SP-$D^{-/-}$ compared with WT mice. *B*, EMSA for NF-κB. Nuclear extracts of AMs were obtained from WT and SP-$D^{-/-}$ mice and NF-κB activation assessed by EMSA. Enhanced DNA binding activities of NF-κB were detected in the nuclear extracts from SP-$D^{-/-}$ compared with those from WT mice. Specific competition with a excess of unlabeled NF-κB oligonucleotide eliminated the NF-κB band. Likewise AP-1 binding activities were enhanced in the nuclear extracts from SP-$D^{-/-}$ mice. *C*, Supershift assay demonstrated bands containing the p50 and p65 subunit, but not c-*Rel*.

appropriate regulation of both oxidant production and inflammatory responses by AMs. SP-D is required for suppression of steady-state NF-κB activation and MMP expression that may contribute to the emphysema characteristic of SP-$D^{-/-}$ mice (4). The increased nuclear translocation and activity of NF-κB seen in the AMs from SP-$D^{-/-}$ mice may influence the heightened inflammatory responses of AMs from these mice during pulmonary infections (5).

Increased oxidative stress in the lungs of SP-$D^{-/-}$ mice was supported by the increased production of ROS by AMs, increased content of oxidized lipid species, reactive carbonyls, and CDCFH fluorescence. However, the mechanism underlying the oxidative stress in the lungs of SP-$D^{-/-}$ mice remains unclear and may relate either to increased oxidant production (4), decreased antioxidant activity, or failure to clear ROS (18). The present studies support the concept that NF-κB activation by AMs was mediated, at least in part, by apocynin- and DPI-sensitive pathways, supporting a role of NADPH oxidase or other oxidases in the process. Recently, NF-κB activation pathway by NADPH oxidase in alcoholic liver injury also was reported (19). However, the specificity of these inhibitors for various oxidases has not been established. Indeed, DPI inhibits a wide range of flavoproteins including NADPH oxidase and complex I within the mitochondrial electron transport chain (20). Therefore, it is possible that pathways other than NADPH oxidase are involved in this process. Recent studies by Bridges et al. (18) also demonstrated that SP-A and SP-D prevented oxidation of unsaturated phospholipids in vitro supporting a direct antioxidant function for these proteins. This activity may be particularly important in the lungs of SP-$D^{-/-}$ mice, wherein concentrations of alveolar lipids are markedly increased and concentrations of SP-A are relatively low (3, 21). Large aggregate surfactant from SP-$D^{-/-}$ mice contained increased lipid peroxide species (M. Yoshida and J. Whitsett, unpublished observations), perhaps reflecting increased oxidant production or decreased oxidant clearance by the lung.

Foamy macrophages are a prominent feature of the lung pathology in SP-$D^{-/-}$ mice (2, 3). The increased oxidant production and

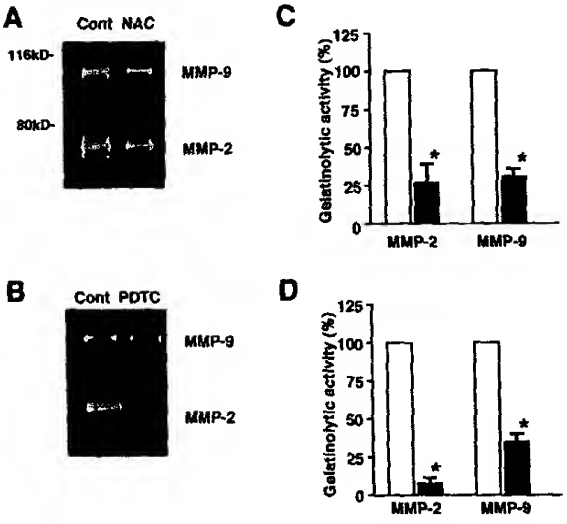

FIGURE 5. Effects of antioxidants on MMP expression by AMs from SP-$D^{-/-}$ mice. AMs were isolated from SP-$D^{-/-}$ mice and treated with 20 mM NAC or 200 μM PDTC. Conditioned medium from the AMs were collected after 24 h of incubation and MMP-2 and -9 activity determined by gelatin zymography. Both NAC (*A*) and PDTC (*B*) inhibited gelatinolytic activities of MMP-2 and -9 in the conditioned medium from SP-$D^{-/-}$ mice. Figures are representative of at least three independent experiments. Densitometric analysis of gelatinolytic activity with (■) or without (□) treatment showed that both NAC (*C*) and PDTC (*D*) significantly inhibited gelatinolytic activities of MMP-2 and -9 in the conditioned medium from SP-$D^{-/-}$ mice. Values were normalized to matched untreated control ± SE; $n = 3$; *, $p < 0.05$.

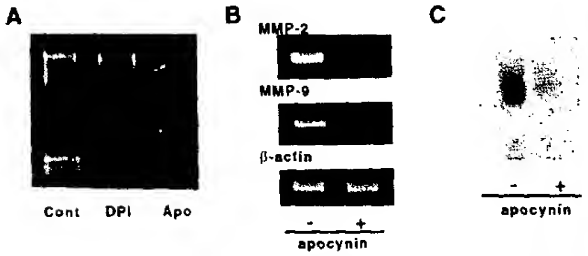

FIGURE 6. NADPH oxidase inhibitors decrease MMP production by AMs from SP-$D^{-/-}$ mice. AMs from SP-$D^{-/-}$ mice were treated with 1 μM DPI and 1 mM apocynin. *A*, Conditioned medium from AMs were analyzed by SDS-PAGE zymography. DPI and apocynin markedly decreased MMP activity. *B*, MMP-2 and -9 mRNA were detected by RT-PCR with specific primers for the cDNA sequences of MMP-2 and -9 as described in *Materials and Methods*. MMP-2 and -9 mRNA were also decreased by the NADPH oxidase inhibitor. *C*, EMSA analysis demonstrated that treatment of apocynin reduced DNA binding activity of NF-κB in AMs isolated from SP-$D^{-/-}$ mice.

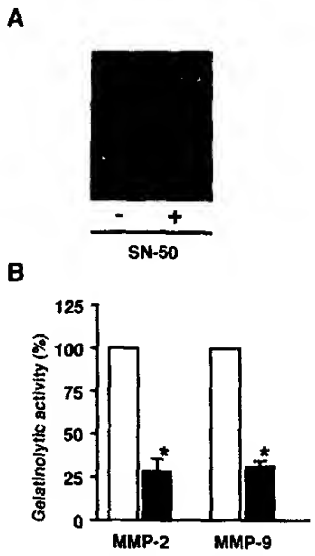

FIGURE 7. SN-50 inhibits MMP expression by AMs from SP-D$^{-/-}$ mice. AMs isolated from SP-D$^{-/-}$ mice were treated with SN-50, a synthetic NF-κB inhibitory peptide. Conditioned medium from the AMs were subjected to zymography in gelatin substrate. SN-50 significantly reduced gelatinolytic activities of MMP-2 and 9 (*A*). The zymogram is representative of three separate experiments. Densitometric analysis of gelatinolytic activity with (■) or without (□) treatment showed that SN-50 inhibited gelatinolytic activities of MMP-2 and 9 in the conditioned medium from SP-D$^{-/-}$ mice (*B*). Values were normalized to matched untreated control ± SE; $n = 3$; *, $p < 0.05$.

foamy AM formation seen in SP-D$^{-/-}$ mice are reminiscent of findings in atheromas, wherein uptake of oxidized lipids by tissue macrophages further induced ROS, and enhanced macrophage activation (22, 23). Although lung lipid concentrations are markedly increased in SP-D$^{-/-}$ mice (3), it is not likely that increased lipid content alone is a sufficient stimulus to generate the activated foamy macrophages. Indeed, similarly increased surfactant lipid concentrations and foamy macrophages were observed in GM-CSF and common β-chain receptor-deficient mice without the increased oxidant production or AM activation seen in the SP-D$^{-/-}$ mice (24, 25). Taken together, the present findings support the concept that SP-D signaling is required for the regulation of oxidant production or clearance of ROS by AMs in the lung. Recent findings that SP-D binds to CD14 via its carbohydrate recognition domain, inhibiting CD14 LPS interactions (26), suggesting a mechanism by which AM activity may be modulated by SP-D. Because LPS-CD14 interactions may also influence NADPH oxidase and NF-κB (19, 27), these pathways may mediate the increased inflammatory responses seen during pulmonary infections in the SP-D$^{-/-}$ mice (5). We also observed that the addition of mouse SP-D (5 μg/ml) in vitro did not reduce MMP production by AMs from SP-D$^{-/-}$ mice (M. Yoshida and J. A. Whitsett, unpublished observations). This finding suggests that direct signaling via SP-D was not sufficient to inhibit MMP production by AMs from SP-D$^{-/-}$ mice in vitro and that activation of AMs may be mediated indirectly by chemical messengers generated in the lungs of SP-D-deficient mice.

SP-D plays an important role in the modulation of pulmonary infection, caused by numerous pathogens (1). SP-D binds to various microorganisms and their products, including Gram-negative and Gram-positive bacteria, respiratory viruses, fungi, and endotoxin. In vitro studies support the role of SP-D in the binding and aggregation of pathogens, enhancing their phagocytosis and killing

properties. Paradoxically, in the presence of some pathogens, o: idant production and killing by AMs in vitro was increased t SP-D, findings that contrast with the marked activation of endo enous oxidant production seen in AMs from the SP-D-deficie mice (4). Thus, SP-D actions on effector cells may be mediated t complex interactions among various receptors that may uniquel recognize the pathogen, SP-D-pathogen complexes, or SP-D. B cause SP-D also binds to various lipid components, includin phosphatidylinositol and glucosylceramide (28, 29), known secon messengers involved in inflammatory responses, SP-D also ma indirectly influence cell signaling by interacting with suc molecules.

The present work demonstrates that the excess ROS generate in the absence of SP-D, activate the redox-sensitive transcriptic factor, NF-B. Thus, SP-D appears to play a central role in th regulation of NF-κB activity in AMs. Because NF-κB regulat numerous proinflammatory response genes expressed by AMs, i cluding IL-1β, TNF-α, IL-6, and MMP-2, and -9 (9), SP-D-d pendent pathways may be important modulators of the gener response of the AM to infection and inflammation. Indeed, in r cent studies, increased production of the cytokines TNF-α, IL- and IL-1β was observed after pulmonary infection by bacteri pathogens in SP-D$^{-/-}$ mice (5), supporting the concept that SP- orchestrates both steady-state and infection-induced proinflamm tory cytokine production by AMs.

In the present studies, SN-50, a selective NF-κB inhibitor, su pressed MMP-2 and -9 production. SN-50 is known to inhibit n clear import of NF-κB, thereby inhibiting its transcriptional acti ity (17). An NF-κB element is present in the promoter region of th MMP-9 gene (10, 30), supporting the concept that SN-50 ma suppress MMP-9 production by blocking NF-κB activity. Hov ever, NF-κB binding sites have not been detected in the promot region of the MMP-2 (12), and it is unclear whether the inhibitor effects of SN-50 on MMP-2 production are regulated by direct indirect effects on MMP-2 transcription. Alternatively, NF-κ may bind to and enhance expression of other transcription factor including AP-1 and p53, that may increase MMP-2 expressio directly or through protein-protein interactions (31, 32). Finall SN-50 shares the nuclear localization sequence that competes f the nuclear import of endogenous NF-κB. The specificity of SN-5 for NF-κB nuclear import has been questioned because other n clear proteins share this nuclear import system (33). Nonetheles the present finding supports the concept that SP-D plays a centr role in the modulation of MMP expression in AMs by influencin NF-κB activity.

The present study demonstrates an oxidant-dependent activatio of NF-κB and enhanced MMP expression by AMs from SP-D$^{-/}$ mice that may be involved in the pathogenesis of emphysema cha acteristic of this model (4). Oxidants derived from air pollutio cigarette smoking, and activated inflammatory cells have been in plicated in the pathogenesis of emphysema in human lung diseas (34). Findings that SP-D concentrations are reduced in lung lavag from smoking individuals (35) and patients with cystic fibros (36) supports a potential role for SP-D in the regulation of oxidan induced lung inflammation.

Acknowledgments

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